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Award Number: W81XWH-04-1-0143

TITLE: Lipoxygenase, angiogenicity, and Prostate Cancer Radioresistance

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REPORT DATE: January 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2007	Annual	01 Jan 04 – 31 Dec 06

4. TITLE AND SUBTITLE
Lipoxygenase, angiogenicity, and Prostate Cancer Radioresistance

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0143

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Southern Illinois University
Springfield IL 62794

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Radiotherapy is a prevalent modality for the treatment of prostate tumor. Although radiation is capable of eradicating localized prostate tumors, nearly 30% of patients treated with potentially curative doses relapse at the sites of irradiation. Therefore, there is an imperative need to improve the success rate of radiotherapy for PCa. This proposal is focused on a role of 12-lipoxygenase (LOX) in modulating the radiation response of PCa cells. 12-LOX catalyzes the formation of 12(S)-hydroxyeicosatetraenoic acid (HETE). Our studies suggest an involvement of 12-LOX in radioresistance of PCa cells. It is our hypothesis that an increase in 12-LOX expression/activity may lead to an increased resistance in tumors to radiation treatment. We also hypothesize that VEGF is an important intermediary for 12-LOX mediated radioresistance in PCa. We intend to define the role of 12-LOX in radioresponse in PCa. 12-LOX will be overexpressed in LNCaP and DU145 cells. Then we will study whether an increase in 12-LOX expression in LNCaP and DU145 cells can enhance their resistance to radiotherapy. We also propose to study whether VEGF is required by 12-LOX to enhance PCa radioresistance through blockade of VEGF activity with a neutralizing antibody. Finally, we will evaluate whether BHPP, a 12-LOX inhibitor, can be used to sensitize prostate tumors to radiotherapy.

15. SUBJECT TERMS
Prostate cancer, radiotherapy, 12-lipoxygenase, apoptosis, angiogenesis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	23	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

Prostate cancer (PCa) is one of most common cancers affecting American men. Radiotherapy is a prevalent modality for the treatment of prostate tumor. Although radiation is capable of eradicating localized prostate tumors, nearly 30% of patients treated with potentially curative doses relapse at the sites of irradiation. Therefore, there is an imperative need to improve the success rate of radiotherapy for PCa.

This proposal is focused on a role of 12-lipoxygenase (LOX) in modulating the radiation response of PCa cells. 12-LOX catalyzes the formation of 12(S)-hydroxyeicosatetraenoic acid (HETE) and it has been implicated in PCa growth and progression. Our studies suggest an involvement of 12-LOX in radioresistance of PCa cells. It is our hypothesis that an increase in 12-LOX expression/activity may lead to an increased resistance in tumors to radiation treatment. Conversely, a downregulation of 12-LOX expression or activity can sensitize PCa cells to radiotherapy. We also hypothesize that VEGF is an important intermediary for 12-LOX mediated radioresistance in PCa. Here we propose to expand our study on the role for 12-LOX in radioresponse in PCa. 12-LOX will be overexpressed in LNCaP and DU145 cells. Then we will study whether an increase in 12-LOX expression in LNCaP and DU145 cells can enhance their resistance to radiotherapy. We also propose to study whether VEGF is required by 12-LOX to enhance PCa radioresistance through blockade of VEGF activity with a neutralizing antibody. Finally, we will evaluate whether BHPP, a 12-LOX inhibitor, can be used to sensitize prostate tumors to radiotherapy. The following specific aims are proposed:

Aim 1. Expand the study on the role of 12-LOX in radioresponse in PCa cells.

Aim 2. Determine whether or not stimulation of VEGF is required by 12-LOX to enhance radioresistance in vitro and in vivo.

Aim 3. Evaluate whether or not 12-LOX inhibitor BHPP can sensitize prostate tumors to radiation in vivo.

BODY OF REPORT

KEY RESEARCH ACCOMPLISHMENT

1 provisional patent application filed
1 review article published
1 research article published
1 review article accepted
2 abstracts published

PROGRESS

Task 1. Expand the study of the role for 12-LOX in radioresponse in prostate cancer cells. Months 1 - 18:

In this aim, the regulation of 12-LOX levels by IR will be studied in a number of prostate cancer cell lines. The radiosensitizing effects of 12-LOX inhibitors in more PCa cell lines and whether 12(S)-HETE can protect them from radiation will be studied. This task has been largely completed, with findings summarized below.

To study whether or not radiation regulates 12-LOX, we subjected LNCaP cells to radiation of different doses and cultured in serum containing media (RPMI1640-10%FBS) for 16 h. LNCaP cells were selected because they express 12-LOX consistently in culture. As shown in **figure 1**, low-dose radiation (200

cGy) increased the protein level of 12-LOX, suggesting that the gene expression of 12-LOX was stimulated by low dose radiation. Interestingly, at higher doses (400 and 1600 cGy), the steady state levels of 12-LOX were reduced. The reduction of 12-LOX level is not due to cell death because we did not notice any significant cell death 16 h after irradiation at doses indicated. The drastic changes in 12-LOX levels as a function of radiation imply that 12-LOX is probably involved in radiation response.

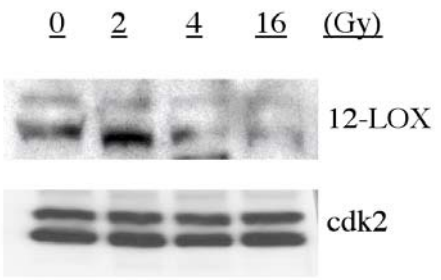

Figure 1*. Effect of radiation on 12-LOX expression in prostate carcinoma LNCaP cells. Note the stimulation of 12-LOX expression by low dose radiation (200 cGy) but at higher doses, 12-LOX expression was reduced (400 cGy and 1600 cGy). The level of cdk2 is included for reference for sample loading.*

To determine whether 12-LOX plays a role in radioresponse of carcinoma cells, we used a panel of PC-3 cell sublines that were stably transfected with an expression construct of platelet-type 12-LOX. The isolated clones had an increased 12-LOX expression and 12(S)-HETE biosynthesis. Next, we examined the effects of increased expression of 12-LOX on colony formation of carcinoma cells after radiation. As shown in **Figure 2**, nL8, a 12-LOX overexpressing clone, presented strong radioresistance when compared to its vector control, neo-α (**Figure 2 A**), as indicated by enhanced clonogenic survival. Regression analysis indicated a significant difference in radioresistance between nL8 and neo-σ ($P < 0.01$) (**Figure 2 B**). The data suggest that increased expression or activity of 12-LOX enhances radioresistance in prostate carcinoma cells.

A **B**

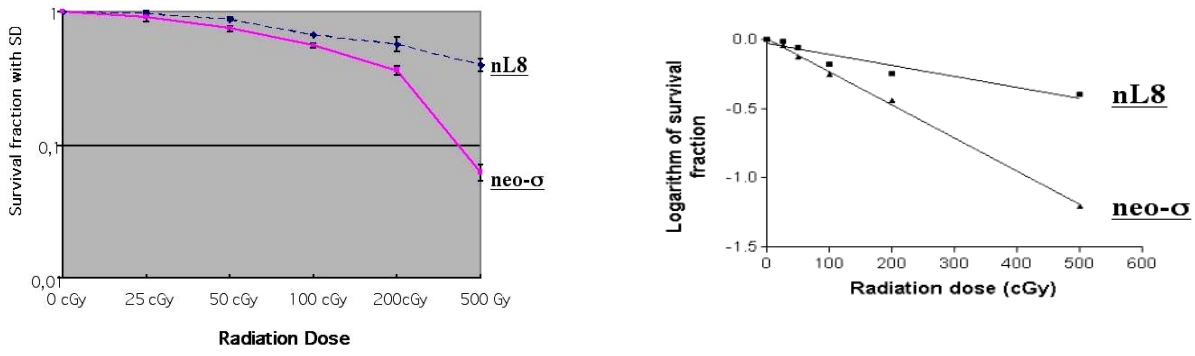

Figure 2*. 12-Lipoxygenase enhances radioresistance of PC-3 cells as indicated by colony formation assay. A, Increased clonogenic survival by enhanced expression of 12-LOX in PC-3 cells. nL8, a 12-LOX overexpressing clone of PC-3 cells; neo-σ, vector control. B, Regression analysis. P< 0.01.*

Next we studied whether baicalein also sensitizes androgen-independent PCa cells to radiation therapy as it did in LNCaP cells. PC-3 cells were treated with 7.5 μM baicalein for two hours before initiation of radiation. As shown in **figure 3 A and B**, baicalein and radiation, when combined, have super

additive or synergistic inhibition on the colony formation of PC3 cells ($P < 0.01$). The data suggest that inhibitor of 12-lipoxygenase also sensitizes androgen independent PC-3 cells to radiation.

A **B**

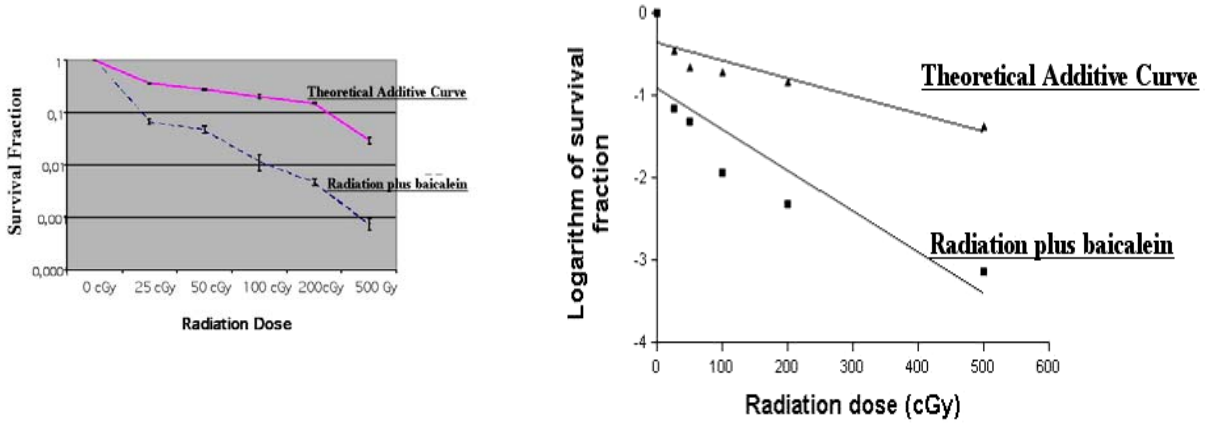

***Figure 3**. Radiosensitization of androgen independent PC-3 cells by baicalein. **A.** 12-LOX inhibitor baicalein sensitizes PC3 cells to radiation as indicated by colony formation assay. **B.** Regression analysis. P = 0.0086.*

Next we examined whether inhibition of 12-LOX can modulate the radioresponse of PCa cells. First we examined the effect of baicalein, a select inhibitor of 12-LOX, on radioresponse of androgen dependent LNCaP cells. We treated LNCaP cells with 7.5 μM baicalein for 2 hrs before initiation of radiation. As shown in **figure 4 A**, baicalein and IR, when combined, have super additive or synergistic inhibitory effect on the colony formation of LNCaP cells. Regression analysis indicates that combined treatment of LNCaP cells with radiation and baicalein has significant super-additive or synergistic effect ($P < 0.05$) (**Figure 4 B**).

A **B**

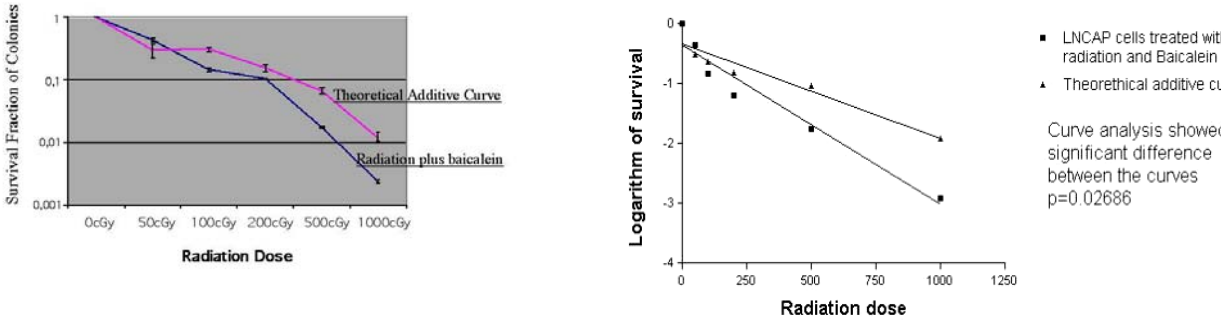

***Figure 4**. Radiosensitization of LNCaP cells by a 12-LOX inhibitor, baicalein. **A.** 12-LOX inhibitor baicalein sensitizes LNCaP cells to IR as indicated by colony formation assay. Refer to the General Method in section D for detailed description of the calculation of theoretical additive curve and other statistical calculation. **B.** Regression analysis. P = 0.02688.*

The main stable arachidonate product of 12-LOX is 12(S)-HETE. To study whether or not 12(S)-HETE modulates radioresistance of carcinoma cells, we treated PC-3 cells with graded levels of baicalein (0, 3, 6, 9, 12, and 15 μM), in the presence or absence of 300 nM of 12(S)-HETE, for 2 h before irradiation (200 cGy). As shown in **Figure 5 A**, baicalein sensitized PC-3 cells to radiation in a dose dependent manner. The radiosensitization of PC-3 cells by baicalein was completely abolished by exogenously added 12(S)-HETE (**Figure 5 A and B**, $P < 0.01$). Therefore, radiosensitization of PC-3 cells by baicalein is dependent on the *absence* of 12(S)-HETE. The results further suggest the involvement of the 12-LOX activity in radioresistance of prostate carcinoma cells.

A *B*

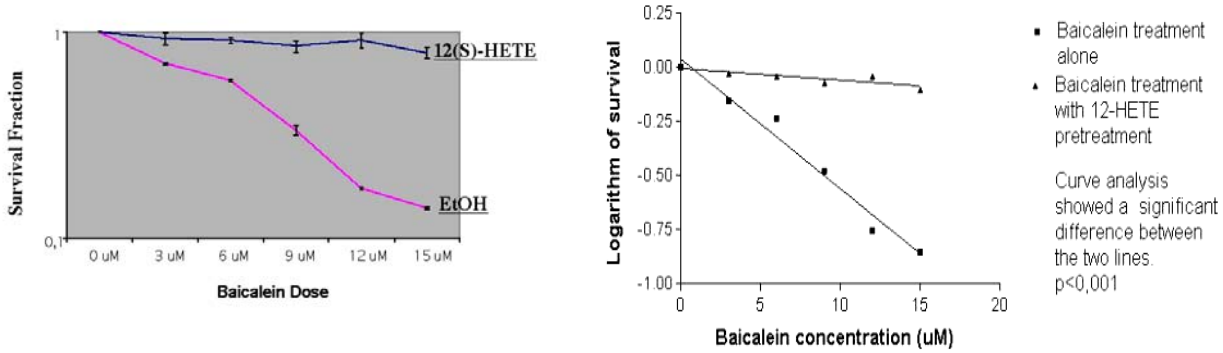

Figure 5*. Radiosensitization of PC-3 cells by baicalein was abolished by exogenously added 12(S)-HETE.* ***A.*** *Attenuation of baicalein radiosensitization of PC-3 cells by 12(S)-HETE as indicated by colony formation assay. The radiation dose used was 200 cGy.* ***B****. Regression analysis. P < 0.001.*

To study whether 12-LOX inhibitors can also sensitize normal prostate epithelial cells to radiation, we treated human normal prostate epithelial cells (purchased from Clonetics, San Diego, CA) with 7.5 μM baicalein 2 h before radiation (800 cGy). The cells are harvested 36 h after radiation for evaluation of apoptosis using a commercial flow cytometric assay kit based on TUNEL staining (APO-DIRECT, Pharmingen, San Diego, CA). We use apoptosis, rather than clonogenic survival, as the end point for potential radiosensitization of normal prostate epithelial cells by 12-LOX inhibitors. The rationale is that unlike prostate cancer cells, normal prostate cells have limited ability to proliferate and form colonies. As shown in **Figure 6** and **Figure 7**, the presence of baicalein did not potentiate radiation-elicited apoptosis either in normal prostate epithelial cells or in human normal skin fibroblast. The lack of radiosensitization by 12-LOX inhibitor in normal prostate epithelial cells may be due to the low or absence of 12-LOX expression (Gao et al., 1995).

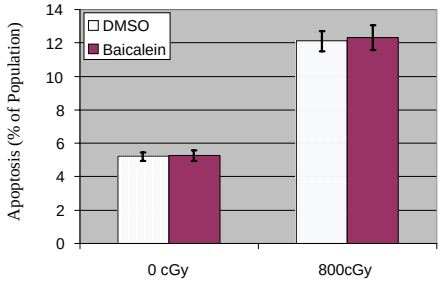

Figure 6*. Lack of radiosensitization of baicalein, a 12-LOX inhibitor, in normal prostate epithelial cells. Note the increase in apoptosis after radiation (800 cGy) and the absence of effect of baicalein treatment on apoptosis, regardless of radiation.*

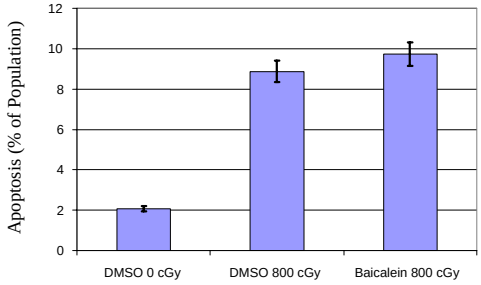

Figure 7*. Lack of radiosensitization of baicalein in normal human skin fibroblast.*

Since 12-LOX inhibitors can induce apoptosis, radiosensitization of tumor cells by baicalein is likely mediated by potentiation of apoptosis. To study this possibility, we evaluated the level of cleaved caspase-3, the activated form of caspase-3. As show in the **figure 8**, combined treatment of A431 cells had highest level of caspase-3 activation.

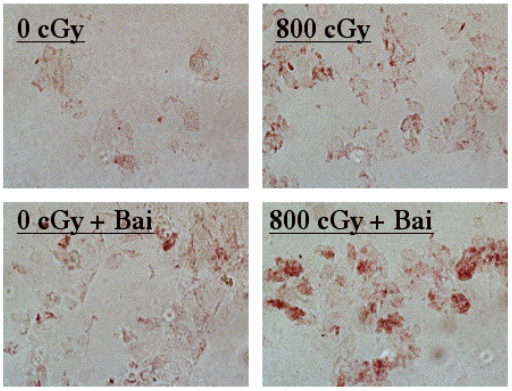

Figure 8. *Levels of cleaved caspase-3 16 h after baicalein and radiation treatment. Cells were fixed and immunostained for cleaved (activated) caspase-3 using standard ABC procedure. Blown staining (dark spots if black and white print) indicates positive staining.*

Critical for apoptotic processes, caspases are cysteine-dependent and sensitive to oxidation, hence, high levels of lipid peroxide from 12-LOX may lead to their inactivation. To study this possibility, we examined whether 12(S)-HpETE can inhibit the activity of Caspase-3, an effector caspase, which can cleave a broad spectrum of cellular targets. As shown in **Figure 9**, 12(S)-HpETE inhibited caspase-3 activity in a dose-dependent manner.

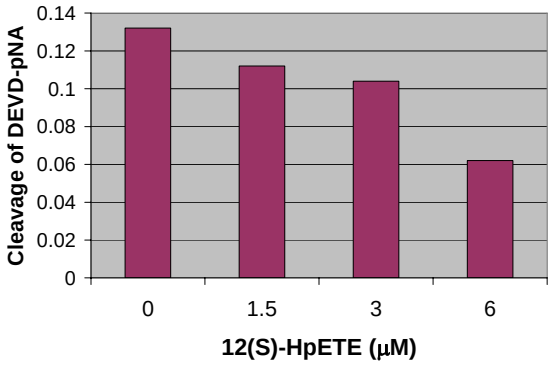

Figure 9*. Inhibition of Caspase-3 activity by 12(S)-HpETE. Active caspase-3 (purchased from Biomol) were incubated with graded levels of 12(S)-HpETE for 10 min, before addition of substrate DEVD-pNA. After further 30 min of incubation, the cleavage of DEVD-pNA was measured at 405 nm and expressed as absolute unit. The results represent two independent experiments.*

Task 2. ***Determine whether or not stimulation of VEGF is required by 12-LOX to enhance radioresistance in vitro and in vivo.***

We will use a VEGF neutralizing antibody to study whether VEGF is required for 12-LOX mediated radioresistance in PC-3 cells. Matrigel implantation model will be used to assess 12-LOX mediated radioresistance in vivo and to study the role of VEGF in this process. This task has been initiated, with the following preliminary findings:

To study whether 12-LOX can regulate VEGF expression, we measured VEGF levels in culture supernatants from 12-LOX transfected PC-3 cells (nL-8 and nL-12) and their vector controls. As shown in **Figure 10**, increased expression of 12-LOX enhanced VEGF expression. Northern blot analysis revealed an increase in the levels of VEGF mRNA in 12-LOX transfected PC-3 cells (nL-2 and nL-8) (**Figure 11)**.

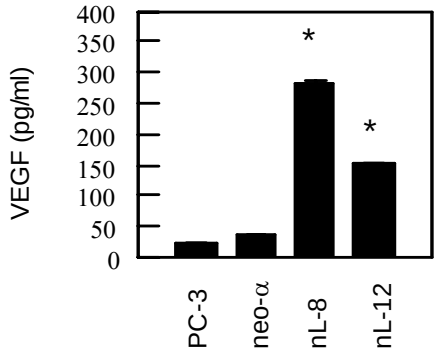

***Figure 10**. Increased VEGF Expression in 12-LOX Transfected PC-3 Cells. *, P < 0.01.*

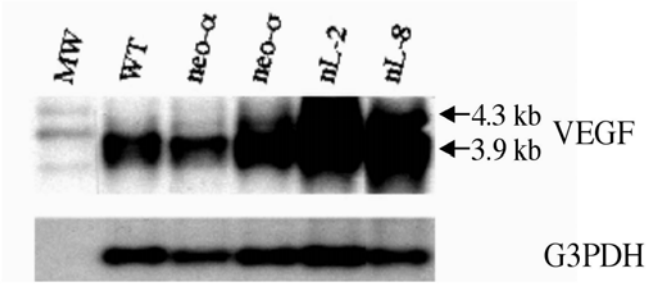

***Figure 11.** Northern Blot Analysis of VEGF mRNA Levels. Poly(A)+RNA were isolated and the 12-LOX mRNA levels were analyzed with labeled VEGF cDNA. The membrane was then stripped and probed for G3PDH as a loading control.*

As part of our effort to study how prostate cancer cells survive clonogenicaly from radiation treatment, we subjected PCa cells to radiation treatment (800 cGy) and the clonogenically survived cells were isolated and propagated. As shown in **Figure 12**, a subline of DU145 cells (DU10a), presented a much higher resistance to subsequent radiation treatment. Similar results were also obtained in PC-3 cells. The data suggest that prostate cancer cells, after surviving potential lethal dose of radiotherapy, become more resistant to subsequent radiation treatment.

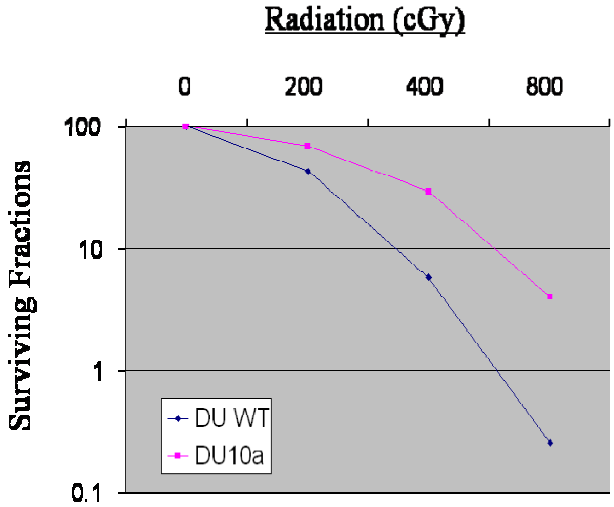

***Figure 12**. Increased radioresistance of DU145 cells after surviving radiotherapy. DU10a are DU145 cells that have survived a potential lethal dose of radiation (800 cGy). DUWT are parental DU145 cells that have survived from sham radiation.*

Next we examined whether there is a change in NF-κB activity, which is known for its role in angiogenesis and tumor growth, in tumor cells surviving radiation treatment. As shown in **Figure 13**, there was a four fold of increase in NF-κB promoter activity in DU10a, as compared to the control, DU145 cells. The data suggest that there is a *sustained* elevation in NF-κB activity in PCa cells that clonogenically survived from radiation treatment.

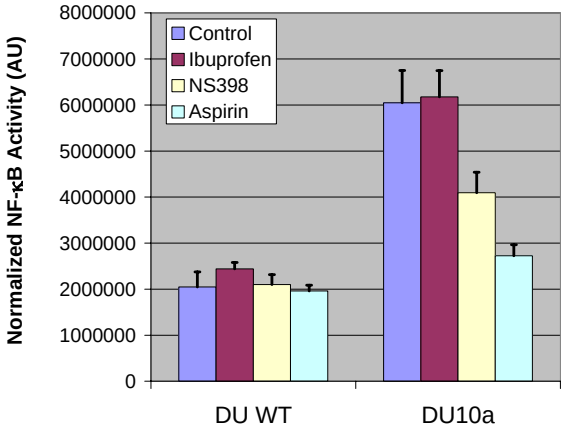

***Figure 13**. Increased NF-κB activity in clonogenically survived DU145 cells (DU10a) and its downregulation by aspirin and NS398. Cells were transfected with a reporter gene construct under NF-kB promoter and treated with ethanol (control), ibuprofen (1 mM), NS398 (20 μM), or aspirin (0.5 mM) for 18 h before luciferase assay. Note the downregulation of NF-κB activity in DU10a cells by NS398 and aspirin.*

Interestingly, the sustained activation of NF-κB in radioresistant DU10a cells was markedly reduced by aspirin treatment (**Figure 13**). The ability of this widely used OTC drug to downregulate the sustained activation of NF-κB in clonogenically survived PCa cells raises an exciting possibility, of using inhibitors of NF-κB, such as sodium salicylates (aspirin), to overcome PCa radioresistance. Studies are ongoing to study whether aspirin can reduce the expression of VEGF and also enhances the efficacy of radiotherapy.

Radiation has been demonstrated to activate NF-κB as part of the immediate early response. We also found that IR can increase NF-κB promoter activities in prostate carcinoma PC-3 and DU145 cells in a dose dependent manner (data not shown) and this activation was associated by a reduction in the level of IκBα (**Figure 14**).

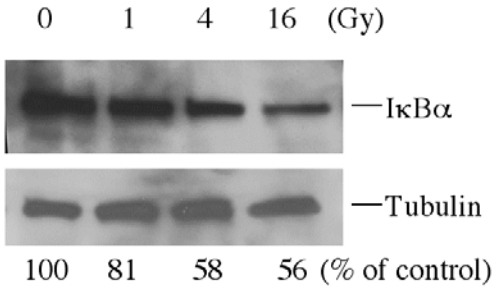

***Figure 14**. Reduction of IκBα levels in prostate carcinoma DU145 cells after IR. Shown here is the level of IκBα level 16 h after IR as revealed by western blot. The level of tubulin was included as a loading control.*

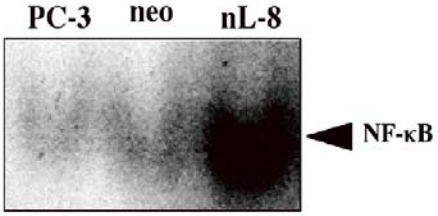

***Figure 15.** Effect of 12-lipoxygenase overexpression on NF-κB activity. EMSA was performed on nuclear extracts of untransfected PC-3, neo, and 12-LOX transfected cells (nL8).Note the increase in NF-κB DNA binding activity in nL8.*

Radiation also rapidly increases the enzymatic activity of 12-LOX. We examined whether or not 12-lipoxygenase regulates NF-κB activity in prostate cancer cells using electomobility shift assays (EMSAs), western blotting for IκBα, and transcriptional activity with luciferase reporter assay. Nuclear protein extracts of 12-LOX transfected cells (nL-8) showed significant constitutive activation of NF-κB compared to the vector control cells (neo-α) or the untransfected PC-3 cells (**Figure 15**).

This activation was further confirmed by the increased transcriptional activity of the luciferase reporter construct, driven by NF-κB, in nL-12 cells (**Figure 16**). This increase in transcriptional activity observed in 12-LOX transfected cells was nearly abolished upon co-transfection of a mutant of IκBα that is resistant to proteolytic degradation purchased from Upstate Biotechonogy (Lake Placid, NJ) (**Figure 16**). Activation of NF-κB involves phosphorylation and eventual degradation of IκB protein before NF-κB could bind to DNA. Western blot analysis of whole cell protein extracts from neo-α, nL-8, and nL-12 cells showed a dramatic decrease in IκBα in nL-8 and nL-12 cells (**Figure 17**, in previous page). These results strongly suggest that overexpression of 12-LOX induces NF-κB activity by a mechanism involving proteolytic degradation of IκBα.

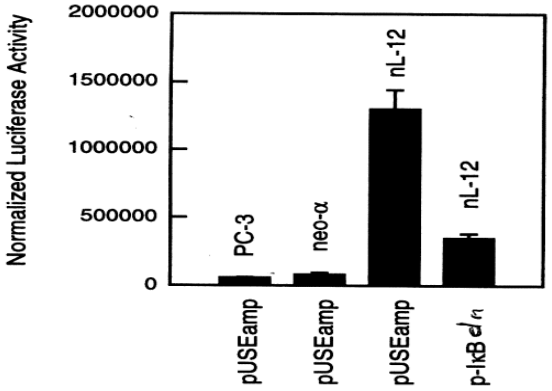

***Figure 16**. Effect of 12-lipoxygenase overexpression on NF-κB activity. The cells (PC-3, neo, and a clone of 12-LOX transfected PC-3, nL-12) were transfected with NF-κB-luciferase and LacZ reporters (pUSEamp) and the reporter activities measured. To elucidate the role of of IκBα in 12-LOX activation of NF-κB, cells were also transfected with a dominant negative mutant IκBα construct (p-IκBdn), or its control vector pUSEamp. The normalized data shown is an average of three experiments with standard deviation. LacZ expression was used for normalization.*

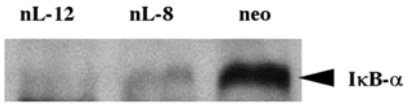

***Figure 17**. Reduction of IκBα levels by overexpression of 12-LOX in PC-3 cells. The levels of IκBα were evaluated by western blot analysis in cell lysates from 12-LOX overexpressing PC-3 cells (nL-8 and nL-12) and their vector control (neo).*

Next we used BHPP, a select inhibitor of 12-LOX (Nie et al., 2000), to study the role of the enzymatic activity of 12-LOX in NF- κB activation. DNA binding activity of NF-κB was greatly decreased upon exposure to 20 μM BHPP for 60 min (**Figure 18**). The results show the participation of the 12-LOX enzymatic activity in NF- κB activation.

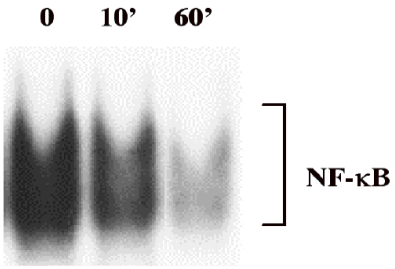

***Figure 18**. Inhibition of NF-κB activation in 12-Lipoxygenase transfected PC-3 cells (nL8) by BHPP, a select 12-LOX inhibitor*

To further study the involvement of 12(S)-HETE in the activation of NF-κB, we evaluated the DNA binding activity of NF-κB when PC-3 cells were treated with 12(S)-HETE. As shown in **figure 19**, 12(S)-HETE can modulate NF-κB DNA binding activity in a dose- and time-dependent manner. Further, the increased NF-κB DNA binding activity was accompanied by the nuclear translocation of NF-κB from cytosol to the nucleus, as revealed by immunocytochemical analysis of the p65 subunit of NF-κB (**Figure 20**).

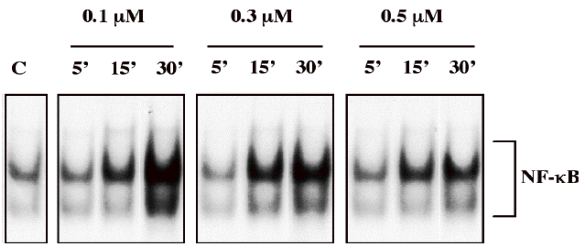

Figure 19. *Effect of 12(S)-HETE on the activation of NF-κB in PC-3 cells. Time and dose dependent activation of NF-κB DNA binding activity by 12(S)-HETE. EMSA was performed on the nuclear extracts of the treated PC-3 cells. The cells were incubated with serum-free RPMI medium containing the amounts of 12(S)-HETE shown for the indicated time and subjected to EMSA.*

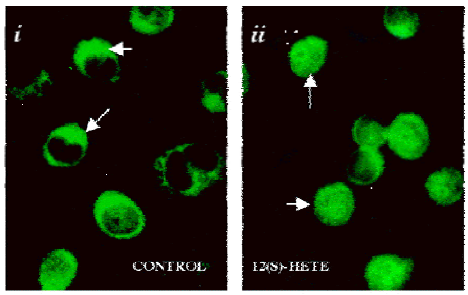

Figure 20: *Immunofluorescent localization of NF-κB in PC-3 cells with and without 12(S)-HETE treatment. Cells were treated with 100 nM 12(S)-HETE or buffer for 10 min and immunostained. Staining was predominantly present in the cytoplasm of untreated control cells (*Left panel, *arrows) and nuclear staining increased considerably in 12(S)-HETE treated cells (*Right panel, *arrows).*

The pro-angiogenic activity of 12-LOX also can play a role in the recurrence of tumor cells at sites of irradiation. The proangiogenic and radioresistant activity of 12-LOX bears a similarity to VEGF, a putative angiogenic factor that is also involved in clonogenic survival of tumor cells after radiation. We thus examined the relationship between 12-LOX and VEGF in PCa cells after radiation. ELISA analysis of the culture supernatants in PC-3 cells 24 hours after irradiation indicated an upregulation of VEGF levels at clinically relevant dosages (Figure, 200 and 400 cGy). Treatment of cells with baicalein (20 μM) abolished the stimulation of VEGF expression by radiation (**Figure 21**), suggesting an important role for 12-LOX in radiation-stimulated VEGF expression.

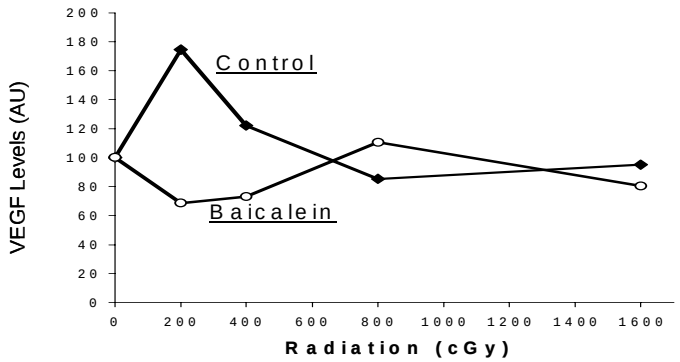

Figure 21*. Stimulation of VEGF production by low dose radiation in PC-3 cells and its dependence on 12-LOX activity.*

Task 3. ***Evaluate whether or not 12-LOX inhibitor BHPP can sensitize prostate tumors to radiation in vivo.*** We will evaluate whether BHPP, a 12-LOX inhibitor, can be used to sensitize xenografted prostate tumors to radiotherapy.

To study whether inhibition of 12-LOX can sensitize prostate tumor to radiation in vivo, we injected 2 X 10^6 12-LOX transfected PC-3 cells (P-LOX), mixed with 0.5 ml 10X diluted Matrigel, s.c. in the middle of the back at 1.5 cm from the tail. Diluted Matrigel was used to promote tumor formation and growth in a more consistent and uniform way. After 4 weeks, palpable tumors reached to a size of 0.2 ~ 0.3 cm in diameter. BHPP (50 mg / kg) or control solvent was injected i.p. into mice 2 hours before radiation treatment of xenografted tumors. For irradiation of xenografted prostate tumors, tumor-bearing mice were immobilized in lead jigs containing cut-out windows of 2cm/2cm for exposure of the s.c. tumors on the lower back. Four jigs at a time were positioned on an aluminum frame mounted on the X-ray machine. Radiation was be delivered to the tumors using a Siemens Stabilipan X ray set operated at 250 kV, 15 mA with 1 mm copper filter at a distance of 28 cm from the start for a single dose of 600 cGy (Sheldon and Hill, 1977; Kjellen et al., 1991). After radiation treatment, the mice were returned to cage and the growth of tumors were monitored closely and tumor sizes measured three times a week. As shown in **Figure 22**, radiation caused a reduction in tumor growth (Rad vs Non-rad). Pretreatment of tumor bearing mice further reduced tumor growth after radiotherapy (Rad vs. BHPP-rad), suggesting that inhibition of 12-LOX may be a promising approach to enhance the efficacy of radiotherapy for prostate tumors in vivo.

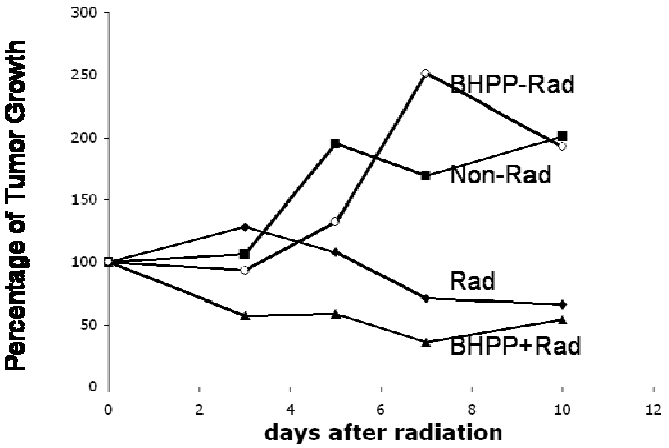

Figure 22. *Effects of 12-LOX inhibitor BHPP on radiation response of prostate tumor in vivo.*

Due to the time lost when the PI moved from Wayne State University to Southern Illinois University School of Medicine, a no-cost extension was requested and approved to continue the animal studies proposed.

SUMMARY/CONCLUSIONS:

Our studies found that 12-LOX promotes the resistance of prostate cancer cells toward radiation treatment. We also found when combined, 12-LOX inhibitors and radiation had synergistic effects in killing

PCa cells and this was accompanied by an increase in the level of the active form of caspase-3. Our studies suggest that 12-LOX inhibitors are promising radiosensitizer and further work need to be done to determine the mechanism of radiosensitization and the efficacy of 12-LOX inhibitors in sensitizing prostate tumors to radiation treatment.

REPORTABLE OUTCOMES

- Two review articles published.
  Nie D, Honn KV.Eicosanoid regulation of angiogenesis in tumors. Semin Thromb Hemost. 2004 30:119-25.
  Nie D. Cyclooxygenases and lipoxygenases in prostate and breast cancer. Frontier in Bioscience 12: 1574-1585, 2007.
- Research article published
  Nie D, Krishnamoorth S, Jin R, Tang K, Chen U, Qiao Y, Zacharek A, Guo Y, Milanini J, Pages G, Honn KV. Mechanisms regulating tumor angiogenesis by 12-lipoxygenase in prostate cancer cells. Journal of Biological Chemistry 281: 18601 – 18609, 2006.
- Abstract published.
  Krishnamoorthy, S., K. R. Maddipati, D. Nie, and K. V. Honn. 12-Lipoxygenase in hypoxia and hypoxia-induced angiogenesis. Proc. Amer. Assoc. Cancer Res. 45: #3591, 2004.
- Abstract published.
  Nie, D., Y. Qiao, A. Zacharek, and K. V. Honn. Blockade of NF-kB sensitizes prostate cancer cells to ionizing radiation. Proc. Amer. Assoc. Cancer Res. 45: #1290, 2004.
- Patent applied. A provisional patent application, entitled “12-Lipoxygenase inhibitors as radiosenstizer for prostate cancer” has been filed.
- Development of animal models: No.

Appendix

1. Research Article published

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 27, pp. 18601–18609, July 7, 2006
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Mechanisms Regulating Tumor Angiogenesis by 12-Lipoxygenase in Prostate Cancer Cells*

Received for publication, February 27, 2006, and in revised form, April 6, 2006 Published, JBC Papers in Press, April 25, 2006, DOI 10.1074/jbc.M601887200

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12-Lipoxygenase utilizes arachidonic acid to synthesize 12(*S*)-hydroperoxyeicosatetraenoic acid, which is converted to the end product 12(*S*)-hydroxyeicosatetraenoic acid, an eicosanoid that promotes tumorigenesis and metastasis. Increased expression of 12-lipoxygenase has been documented in a number of carcinomas. When overexpressed in human prostate or breast cancer, 12-lipoxygenase promotes tumor angiogenesis and growth *in vivo*. The present study was undertaken to delineate the mechanisms by which 12-lipoxygenase enhances angiogenesis. Herein we report that nordihydroguaiaretic acid, a pan inhibitor of lipoxygenases and baicalein, a selective inhibitor of 12-lipoxygenase, reduced VEGF expression in human prostate cancer PC-3 cells. Overexpression of 12-lipoxygenase in PC-3 cells resulted in a 3-fold increase in VEGF protein level when compared with vector control cells. An increase in PI 3-kinase activity was found in 12-LOX-transfected PC-3 cells and inhibition of PI 3-kinase by LY294002 significantly reduced VEGF expression. Northern blot and real time PCR analyses revealed an elevated VEGF transcript level in PC-3 cells transfected with a 12-lipoxygenase expression construct. Using a VEGF promoter luciferase construct (−1176/+54), we found a 10-fold increase in VEGF promoter activity in 12-lipoxygenase-transfected PC-3 cells. The region located between −88 and −66 of the VEGF promoter was identified as 12-lipoxygenase responsive using VEGF promoter-based luciferase assays. Further analysis with mutant constructs indicated Sp1 as a transcription factor required for 12-lipoxygenase stimulation of VEGF. Neutralization of VEGF by a function-blocking antibody significantly decreased the ability of 12-lipoxygenase-transfected PC-3 cells to stimulate endothelial cell migration, suggesting VEGF as an important effector for 12-lipoxygenase-mediated stimulation of tumor angiogenesis.

Arachidonic acid metabolism is catalyzed by two major groups of enzymes, cyclooxygenases (COX)[3] and lipoxygenases (1). Metabolites of these pathways are involved in various steps of carcinogenesis by modulation of cell signaling cascades and mitogenic pathways (2, 3). Several lines of evidence indicate 12-lipoxygenase (12-LOX) as a key regulator of human cancer development. Overexpression of 12-LOX has been detected in a variety of tumors including breast, renal, pancreatic, and prostate cancer (4–6). In addition urinary levels of 12-hydroxyeicosatetraenoic acid (12-HETE), the metabolite produced by 12-LOX, in prostate cancer patients are significantly elevated when compared with normal individuals and removal of the prostate gland results in a significant decrease in the urinary concentration of this eicosanoid (7) In contrast, other HETEs (*i.e.* 5- and 15-HETE), although detected remain unchanged following radical prostatectomy (7). Approximately 38% of the 138 prostate cancer patients studied exhibited an elevated expression of 12-LOX at the mRNA level in prostate tumor tissues compared with matched normal tissues. This elevated 12-LOX mRNA expression was found to have a positive correlation with advanced stage and poor differentiation of prostate cancer (6). In accordance with these findings, 12-LOX also plays a significant role in tumor cell survival and apoptosis (8). Inhibition of 12-LOX activity in prostate cancer cells results in cell cycle arrest and induction of apoptosis (9) and overexpression of 12-LOX in PC3 cells leads to an increase in cell survival by the up-regulation of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins (10). Tumor xenografts in mice, generated by the injection of the 12-LOX-overexpressing cells were more angiogenic compared with the tumors formed by the empty vector control cells. These tumors demonstrated a higher microvessel density and these vessels were better organized compared with the controls. Conditioned medium from these cells in culture as well as 12(*S*)-HETE showed an enhanced capability in stimulating endothelial cell migration compared with medium from vector control cells (11).

The acquisition of the angiogenic phenotype by cancer cells is a pivotal point in the progression and metastasis of solid tumors. Angiogenesis, a complex process involving the formation of new blood vessels from the existing vasculature, is a seminal event in the field of tumor biology (12). Angiogenesis is mediated by a complex interplay of a variety of factors which include tumor and endothelial cells, pro-angiogenic factors *i.e.* vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), proteolytic enzymes, for example MMPs, cell surface molecules, for example integrins and many other factors (13). In addition, anti-angiogenic factors also play a role in regulating blood vessel formation and the balance between the pro- and anti-angiogenic factors determines the rate of formation of the neovasculature. Pro-angiogenic gene expression is activated by a multitude of factors, which include physiological stimuli such as hypoxia, oncogene activation, and tumor suppressor mutations (14). Several experimental observations have proved that VEGF is a potent tumor angiogenesis factor. Agents that target VEGF and its signaling pathways inhibit tumor growth and propagation in various experimental models (15). The promoter region of the *VEGF* gene has binding sites for transcription fac-

* This work was supported by National Institutes of Health Grant CA-29997 (to K. V. H.), United States Army Prostate Cancer Research Program DAMD 17-98-1-8502 (to K. V. H.), and United States Department of Defense Prostate Cancer Research Program New Investigator Award No. W81XWH-04-1-0143 (to D. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: COX, cyclooxygenase; LOX, lipoxygenase; HPETE, hydroperoxyeicosatetranoic acid; HETE, hydroxyeicosatetraenoic acids; NDGA, nordihydroguaiaretic acid; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL-8, interleukin 8; PKC, protein kinase C; FBS, fetal bovine serum; ELISA, enzyme-linked immunoassay; PI, phosphatidylinositol; gal, galactosidase.

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tors like Sp1/Sp3, AP-2, Egr-1, STAT-3, and HIF-1 (16). Of these factors, HIF-1 is responsible for transcriptional regulation under hypoxic conditions (17). VEGF expression also depends on post-transcriptional mechanisms, chiefly the stabilization of the VEGF mRNA, which is a major event under hypoxia (18).

The mechanisms underlying the elevated angiogenic response exhibited by 12-LOX-overexpressing PC-3 cells is unknown. Therefore, the present study was undertaken to delineate the molecular mechanisms underlying the enhanced angiogenic response mediated by 12-LOX in prostate tumors. These studies reveal that 12-LOX-overexpressing PC-3 cells secrete high concentrations of VEGF and demonstrate that 12-LOX mediates an increase in VEGF promoter activity and secretion through the Sp-1 and AP2 transcription factors in a PI 3-kinase/Akt-dependent fashion. Our data provide a novel insight into the regulation of angiogenesis by eicosanoids.

EXPERIMENTAL PROCEDURES

Plasmids—The VPF/VEGF reporter construct, along with mutant constructs in the region between −88 and −66 of the VEGF promoter, were previously described (19). A LacZ expression construct was purchased from Stratagene (La Jolla, CA).

Cell Culture and Generation of Stable Transfectants—PC-3 and Du145 cells were purchased from ATCC maintained in RPMI 1640 supplemented with 10% FBS (Invitrogen) in a humidified incubator supplied with 5% CO_2 and air at 37 °C. PC-3 cells were plated in 6-well plates and incubated overnight in medium containing 10% fetal bovine serum. The cells were transfected with a platelet-type 12-LOX expression vector (gift from Dr. Colin Funk) or the empty vector (pcDNA 3.1(+)) (Invitrogen), using Geneporter Reagent (Genetherapy Systems) according to the manufacturer's instructions. After 48 h, cells were placed in selection medium containing G418 (Invitrogen). Clones expressing high levels of 12-LOX were isolated, and the protein levels of 12-LOX-expressing clones, named nL2, nL8, and nL12 were compared with the empty vector control cells (neo) and wild-type PC-3 cells. These cells were characterized in terms of their metabolic activity to produce higher levels of 12(*S*)-HETE compared with the vector control cells (neo) as described previously (11). 12-LOX-transfected PC-3 cells (nL2, nL8, and nL12), and vector controls (neo-α, neo-σ, and neo-θ) were maintained in RPMI 1640 with 10% FBS in the presence of 0.2 mg/ml of G418 (Geneticin). Prior to use cells overexpressing 12-LOX were routinely confirmed for 12-LOX overexpression by Western blot with antisera against 12-LOX (Oxford Biomedical Research, Oxford, MI). HUVECs were purchased from Clonetics (San Diego, CA), maintained in EGM-2 and used between passage numbers 2 and 9.

Assays for Cytokine Measurement—For treatment of cells with inhibitors, 2×10^6 prostate cancer cells were plated in 35-mm wells using 6-well culture plates and cultured in RPMI 1640 containing 10% FBS. Cells cultured in dishes were treated with various inhibitors as indicated in the text for 24 or 48 h. For each treatment, three wells were used. The culture media were then harvested for determination of VEGF levels or other cytokines using ELISA immunoassay kits from R & D Systems. For treatment of PC-3 cells with 12(*S*)-HETE, PC-3 cells were cultured in serum-free RPMI 1640 with 0.5% lipid free bovine serum albumin and then treated with escalating concentrations of 12(*S*)-HETE (Cayman Chemicals) for 48 h before harvesting the culture medium. VEGF165, the secreted predominant VEGF isoform in humans, derived from alternative splicing (20), bFGF, and IL-8 were measured in conditioned cell culture medium using ELISA immunoassay kits according to the manufacturer's instruction.

Isolation of RNA, Northern Blotting, and Real Time PCR Analyses—Poly(A) mRNAs were isolated from wild-type PC-3, neo-α, neo-σ, nL2, and nL8 cells using an oligotex mRNA kit (Qiagen, Valencia, CA). For Northern blot analysis, aliquots of poly(A) mRNA (2.5 μg) were subjected to electrophoresis on a 1% agarose formaldehyde gel and transferred to Duralon-UV membranes (Stratagene). The membranes were subjected to UV cross-linking in a Stratagene Crosslinker (Stratagene). The VEGF cDNA was kindly provided by Dr. Shin Ohnishi (21). Membranes containing the transferred RNAs were prehybridized with QuikHyb solution (Stratagene) at 65 °C for 1 h and then hybridized with denatured [$^{32}P_i$]VEGF cDNA and salmon sperm DNA at 65 °C for 2 h. Membranes were washed with 2× standard saline citrate (SSC)/0.1% SDS for 15 min at room temperature twice and then rinsed with 0.1× SSC/0.1% SDS for 5 min at 65 °C. Radioactivity from the membranes was monitored with a Geiger counter until the background was at low levels, and the wet membranes were wrapped with plastic wrap and exposed to Kodak XAR-5 film for 24 h at −80 °C. The membranes were reprobed with the ^{32}P-labeled human glyceraldehyde-3-phosphate dehydrogenase (Clontech Laboratory Inc, Palo Alto, CA) as a loading control for mRNA levels in each sample.

For Real Time PCR (RT-PCR) analysis, total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer's protocol with an additional step of membrane DNase treatment (using an Rnase-free DNase from Qiagen) to remove possible genomic DNA contamination. Total RNA was reverse-transcribed to cDNA using the SuperScript III first-strand synthesis system (Invitrogen) with oligo$(dT)_{20}$ primer. For real time detection of target gene expression, TaqMan® Gene Expression assays (Applied Biosystems) were used. PCR was performed using Applied Biosystems 7000 Real-Time PCR System in a total reaction mixture of 25 μl containing 150 ng of cDNA, 1× ABI TaqMan PCR Master mix and 1.25 μl of probe/primers mixture (TaqMan®® Gene Expression assays). After denaturation at 95 °C for 10 min, 40 cycles were performed at 95 °C for 10 s, 60 °C for 1 min (universal cycling conditions). Relative quantification was calculated using the comparative C_T method also known as the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta C_{t\ sample} - \Delta C_{t\ reference}$) as described previously (22). Lower ΔC_T values and lower $\Delta\Delta C_T$ reflect a relatively higher amount of gene transcript.

Transient Transfection to Measure VEGF Promoter Activities—Cells were plated at 2×10^5 cells per 35-mm dish 2 days before transfection with the −1176/+54 VEGF promoter-luciferase construct, or other constructs as indicated, and a LacZ expression plasmid using GenePorter transfection reagent following the manufacturer's protocol (Gene Therapy System). Cells were harvested for luciferase assay 36 h after transfection. Luciferase activity was measured using the luciferase assay kit (Promega, Madison, WI). In all co-transfection experiments, transfection efficiency was normalized by assaying β-galactosidase activity using the β-galactosidase assay kit (Invitrogen).

Immunoblotting—Immunoblotting was used to evaluate the level of 12-LOX protein expression, and phosphorylation of Akt. Semiconfluent (70–80%) cells were rinsed with ice-cold phosphate-buffered saline, scraped into lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM phenylmethylsulfonyl 4-fluoride, 0.5 mM leupeptin, 0.15 mM pepstatin A, 1 mM dithiothreitol, and 1% Nonidet P-40. Protein concentration was measured using the BCA protein assay kit (Pierce) and 30-μg protein samples were mixed with 2× SDS sample buffer and subjected to electrophoresis in pre-made 4–20% gradient SDS-PAGE gels and transferred to a nitrocellulose membrane. After transfer, the membrane was blocked with TBS-T containing 5% low fat milk for 60 min, incubated with the primary antibody at the appropriate

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dilution (rabbit anti-Akt and anti-P-473 Ser Akt antibodies 1:1000; rabbit anti-12-LOX, 1:1000; mouse anti-β-actin 1:2500) in TBS-T with 5% low fat milk at 4 °C overnight. Polyclonal antibodies against Akt and phospho-Akt were purchased from Cell Signaling Inc. Monoclonal anti-β-actin antibody was purchased from Chemicon. After overnight incubation with primary antibody, the membrane was washed (3×) with TBS-T and probed with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (1:2000) for 1 h at room temperature and washed (3×) with TBS-T for a total of 15 min. The immunoblots were visualized by enhanced chemiluminescence (Amersham Biosciences).

In Vitro PI 3-Kinase Assay—In vitro phosphorylation of phosphatidylinositols was carried out with PY20 antibody (Transduction Laboratory) for the detection of immunoprecipitated PI 3-kinase essentially as previously described (23). Prostate cancer cells were harvested with phosphate-buffered saline containing 1% Nonidet P-40, 1 mM dithiothreitol, 200 μM sodium vanadate, 100 μM 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin (lysis buffer). Soluble cell lysates were collected after microcentrifugation at 18,000 × *g* for 5 min. The protein concentration of cell lysates was adjusted to 0.8–1 μg/μl with lysis buffer and PY20 mAb (5 μg) was added to 500 μl of the cell lysate. The mixture was incubated with gentle rocking at 4 °C overnight, and then 10 mg of protein A-Sepharose (Amersham Biosciences) were added, and the incubation was continued for another 2 h. The immunoprecipitates were washed (3×) with the aforementioned lysis buffer, twice with 0.1 M Tris/HCl, pH 7.5, containing 0.5 M LiCl, and 10 μM sodium vanadate, and twice with 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 10 μM sodium vanadate, and 1 mM EDTA. Then 50 μl of kinase buffer (10 mM HEPES (pH 7.0), 0.1 mM EGTA, 25 mM $MgCl_2$, 100 μM ATP, 10 μCi of [^{32}P]ATP, 10 μg/sample PtdIns) was added to each sample and incubated at room temperature for 10 min. The assay was terminated by adding 60 μl of 2 μM HCl and 160 μl of chloroform/methanol (1:1)/sample, and followed by vortexing and centrifugation. The chloroform phase, which contained the inositides was removed and applied to Silica gel 60 thin layer chromatography (TLC) sheets (Merck). TLC was developed with chloroform/methanol/24% ammonium hydroxide/H_2O (90:90:9:19). After drying, spots were located by autoradiography and compared with standards.

Endothelial Cell Migration Assay—The migration assay was performed to assess the angiogenic potential of tumor cells using 96-well Chemo-TX invasion plate (Neuroprobe). HUVECs were harvested by trypsinization in the presence of EDTA and suspended in EBM-2 with 2% FBS at density of 5×10^5 cells/ml. To study whether PC-3 cells secrete chemotactic factors which stimulate endothelial cell migration, the conditioned medium from 12-LOX-transfected PC-3 cells and their vector control cells were concentrated (6×) using Centricon-10. The concentrated culture supernatants were then diluted (10×) with EBM-2% FBS and added in the bottom chamber of the well in the presence of neutralizing antibodies against VEGF-A, bFGF, or IL-8. Migration was initiated by adding cells (20 μl) to the upper chamber. After 12–18 h, the cells on the upper side of membrane were removed using Kimwipes, and the plate was fixed and stained. Cells migrated were enumerated in a double blind approach. Usually 5 fields (×100) representing two perpendicular cross-lines of each membrane were counted. For each treatment, at least 6 chambers were used.

RESULTS

Reduction of VEGF Expression by 12-LOX Inhibitors—Previous studies clearly have demonstrated that 12-LOX is expressed in prostate carcinoma cells (23) and the level of 12-LOX mRNA exhibits a positive correlation with tumor grade and stage (6). In addition, PC-3 cells over-

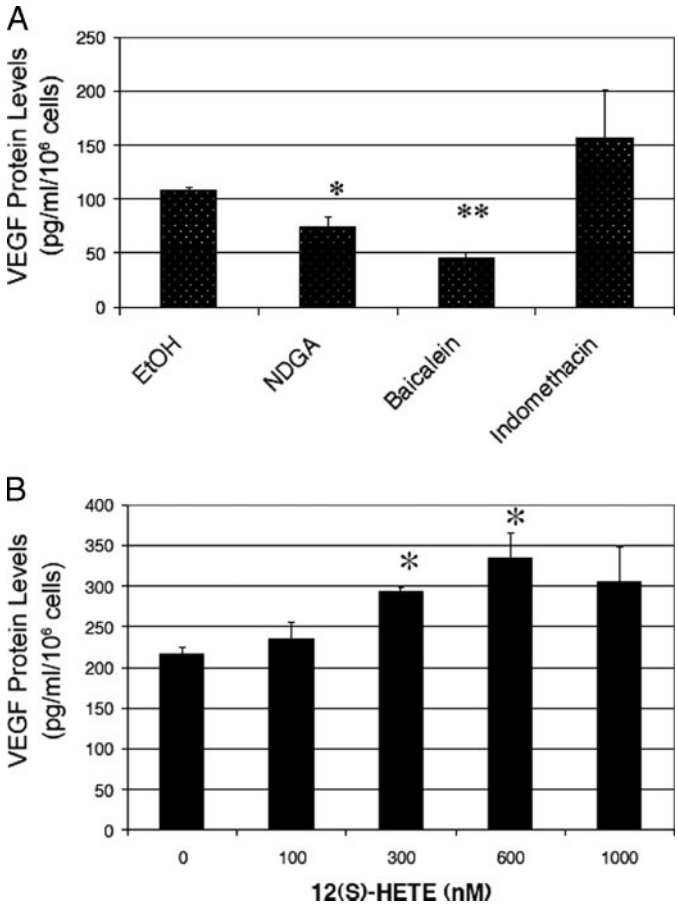

FIGURE 1. **Modulation of VEGF expression by LOX.** *A*, reduction of VEGF protein levels by LOX inhibitors. The level of VEGF protein secreted from PC-3 cells treated with NDGA (50 μM), baicalein (10 μM), or indomethacin (50 μM) were determined using ELISA of the tumor cell-conditioned medium and normalized to the cell number. Values are means ± S.D. of three culture dishes. *, $p < 0.05$ and **, $p < 0.01$. Shown here is a representative result from three independent experiments. *B*, stimulation of VEGF secretion in PC-3 cells by 12(*S*)-HETE. PC-3 cells were treated with escalating concentrations of 12(*S*)-HETE as indicated for 48 h, and the protein levels of VEGF in the culture medium were measured using ELISA and normalized to the cell number. Values are means ± S.D. of three culture dishes. *, $p < 0.05$. Shown here is a representative from three independent experiments.

expressing 12-LOX generate highly angiogenic tumors in mice (11). A similar observation, regarding the proangiogenic role for 12-LOX, also has been demonstrated in MCF-7 breast cancer cells (24). Considering these findings, we examined whether 12-LOX regulates the production of VEGF, a putative angiogenic factor in prostate carcinoma. PC-3 cells were treated with either NDGA (50 μM), a pan-lipoxygense inhibitor or baicalein (10 μM), a specific platelet-type 12-LOX inhibitor for 24 h and the culture medium tested for VEGF protein levels using enzyme immunoassay. As shown in Fig. 1*A*, both NDGA and baicalein significantly decreased VEGF secretion ($p < 0.05$ and $p < 0.01$, respectively) whereas a COX inhibitor, indomethacin (50 μM), increased VEGF levels but not at statistically significant levels. The results suggest that 12-LOX may play a role in enhancing VEGF secretion from prostate cancer cells. 12-LOX converts arachidonic acid to 12(*S*)-HPETE, which is converted to 12(*S*)-HETE. To study whether 12(*S*)-HETE can regulate VEGF expression, PC-3 cells were treated with graded levels of 12(*S*)-HETE, and the protein levels of VEGF in culture medium were assayed by ELISA, normalized to the cell number. As shown in Fig. 1*B*, 12(*S*)-HETE treatment stimulated VEGF secretion from PC-3 cells within 48 h of treatment with optimal doses of 300–600 nM. The results suggest that exogenously added 12(*S*)-HETE regulates the production of VEGF in prostate cancer cells.

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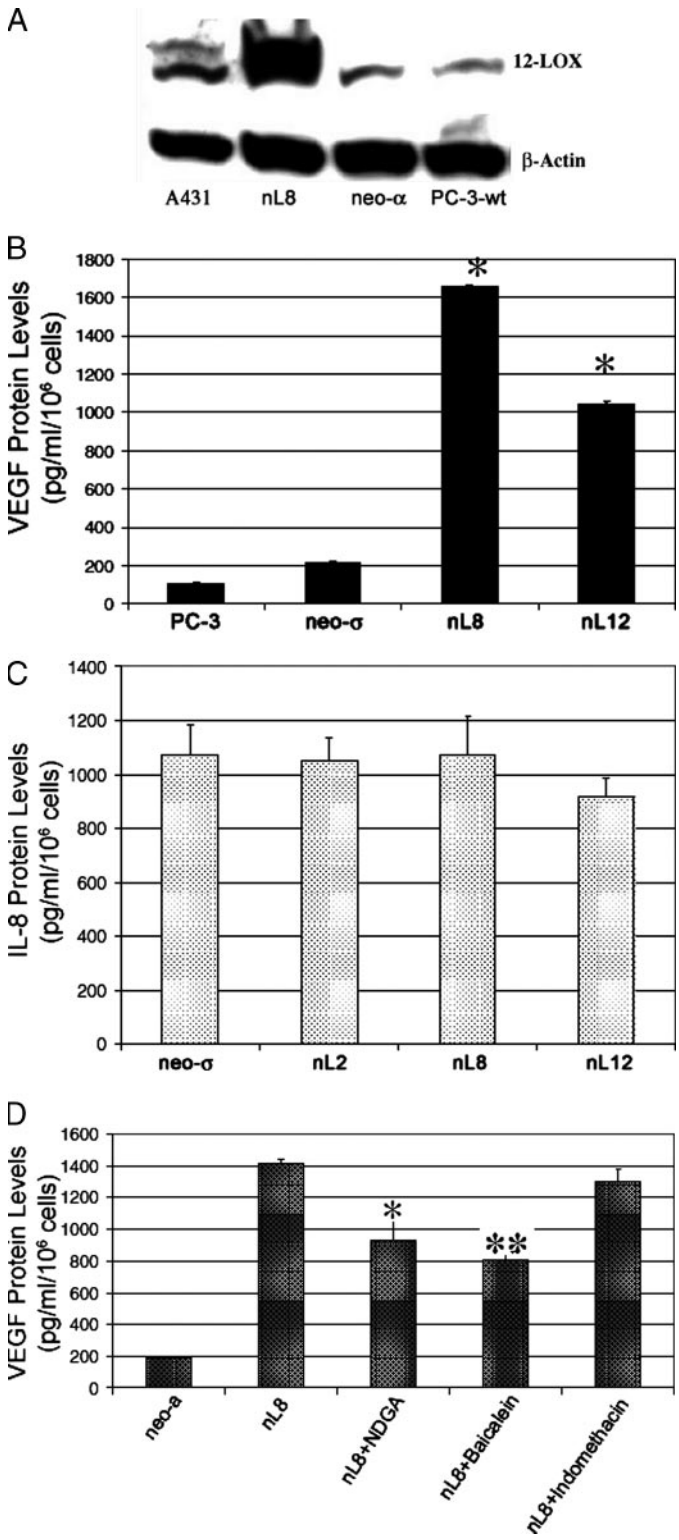

FIGURE 2. **Increased VEGF protein levels in PC-3 cells as result of increased 12-LOX expression.** *A*, Western blot analysis of 12-LOX transfectant (nL8) compared with the vector control (neo α) and wild-type PC3 cells. A431 cell lysates were used as a positive control. β-Actin was used as the loading control. *B*, increased VEGF production in PC-3 cells as result of 12-LOX overexpression. The levels of VEGF in the conditioned medium from 12-LOX-transfected PC-3 cells (nL8 and nL12), vector control cells (neo-σ), and PC-3 parental cells were determined using ELISA and normalized to the cell number. Values are means ± S.D. of three culture dishes. *, $p < 0.01$. Shown here is a representative result from three independent experiments. *C*, levels of IL-8 expression in PC-3 cells in 12-LOX-transfected PC-3 cells (nL2, nL8, and nL12) and their vector control cells (neo-σ) as measured by ELISA of the culture medium and normalization to cell number. Note the lack of significant change in IL-8 production in PC-3 cells as result of 12-LOX expression. Values are means ± S.D. of three culture dishes. *D*, down-regulation of VEGF production in nL8 by NDGA or baicalein but not by indomethacin. The levels of VEGF protein in the conditioned medium from nL8 cells treated with NDGA (50 μM), baicalein (10 μM), or indomethacin (50 μM) were determined using ELISA. Note the reduction of VEGF levels by NDGA or baicalein, but not by indomethacin. *, $p < 0.05$; **, $p < 0.01$, when compared with the control.

Increased VEGF Secretion in 12-LOX-overexpressing PC-3 Cells—Previous studies demonstrated that tumors from 12-LOX-overexpressing PC-3 cells have greater angiogenic response compared with the control population (11). To investigate the mechanism of 12-LOX-mediated enhancement of angiogenesis, we used PC-3 cells stably transfected with the platelet-type 12-LOX expression vector. These 12-LOX-transfected PC-3 cells were found to express higher levels of 12-LOX compared with their vector control (Fig. 2*A*). The cells were characterized for the presence of higher levels of 12(*S*)-HETE production as described previously (11). ELISA for VEGF in the tumor-conditioned medium collected after 24 h of culture, demonstrated a higher secretion of VEGF by the nL8 and nL12 cells compared with empty vector-transfected cells (neo-σ) and wild-type PC-3 cells (Fig. 2*B*). No significant changes in IL-8 levels were found in culture medium from 12-LOX-transfected PC-3 cells compared with the control cells (Fig. 2*C*). We also did not detect bFGF in culture medium from PC-3 or any other transfectants (data not shown). These findings suggest a selective modulation of VEGF secretion by 12-LOX in prostate cancer cells.

To verify that the observed increase in VEGF secretion is indeed an effect of 12-LOX enzymatic activity, we treated nL8 cells with NDGA (50 μM), baicalein (10 μM), or a COX inhibitor indomethacin (50 μM). Conditioned medium was collected from cells treated with each inhibitor, and the levels of VEGF were assayed by ELISA and normalized to cell number. We observed that NDGA or baicalein decreased VEGF secretion from nL8 cells compared with untreated nL8 cells (Fig. 2*D*). Also, we observed that indomethacin, a COX inhibitor, did not effect VEGF secretion from nL8 cells (Fig. 2*D*). The reduction of VEGF secretion in 12-LOX-overexpressing cells upon treatment with inhibitors suggests that the enzymatic activity of 12-LOX is required for VEGF secretion in these cells. These findings provide an explanation for our previous observation (11) of a higher angiogenic response in tumors formed from 12-LOX-overexpressing cells.

Transcriptional Regulation of VEGF Expression by 12-LOX—Regulation of *VEGF* gene expression occurs at transcriptional, post-transcriptional, and/or translational levels (16). To determine whether the increased secretion of VEGF in 12-LOX-overexpressing PC-3 cells results from an increase in the transcriptional rate of the *VEGF* gene, we performed Northern blot and Real Time PCR analyses of VEGF transcription in these cells. Northern blot analysis revealed a substantial increase in the steady state levels of VEGF mRNA in 12-LOX-transfected PC-3 cells (nL2 and nL8) when compared with the neo vector control cells (Fig. 3). Similarly, RT-PCR analysis also demonstrated that nL8 cells have higher levels of VEGF mRNA compared with the neo cells. The ΔCT values for VEGF mRNA were 8.3 and 9.14 in nL8 and neo cells, respectively, with a p value of 0.035. From these experiments, it is evident that 12-LOX-mediated increase in VEGF secretion involves an increase in the steady state level of VEGF mRNA in the 12-LOX-overexpressing cells.

An increase in VEGF mRNA levels may be attributed to either an increase in transcriptional rate or an increase in the stability of the VEGF mRNA (16, 18, 25) or both. To identify the influence of 12-LOX on the transcription of the *VEGF* gene, we employed promoter luciferase assays. The p1176-luc vector, a VEGF promoter luciferase vector used in these studies, consists of the VEGF promoter region (−1176/+54) fused upstream of a luciferase gene in a PGL2 vector (19). nL8,

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nL12, and neo cells were transfected with the p1176-luc vector along with the LacZ plasmid to normalize transfection efficiency. Luciferase assay was performed on the lysed cells, and the values were normalized to β-gal enzyme activity measured using a β-gal assay kit. The assay revealed that there was an ~10-fold increase in VEGF promoter activity in 12-LOX-overexpressing nL8 and nL12 cells compared with the empty vector control cells and the wild-type PC3 cells (Fig. 4*A*). These findings suggest that the observed increase in VEGF mRNA levels and secretion is a result of an increased transcriptional activity of the *VEGF* gene in 12-LOX-transfected PC-3 cells.

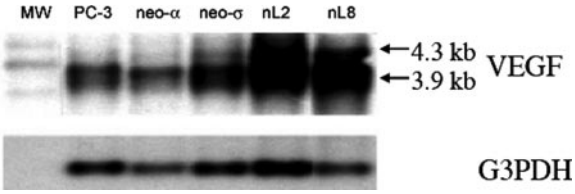

FIGURE 3. **Regulation of VEGF gene expression by 12-LOX.** Northern blot analysis of VEGF mRNA levels in 12-LOX-transfected PC-3 cells (nL2 and nL8), their vector controls (neo-α and neo-σ), and PC-3 parental cells. Poly(A)$^+$ RNA were isolated from 80~90% confluent cultures, subjected to electrophoresis on a 1% agarose formaldehyde gel, transferred to Duralon-UV membrane, and probed with ^{32}P-labeled VEGF or G3PDH cDNA. Shown here is a typical result from two experiments. Note the increase in the levels of VEGF mRNA (3.8 and 4.3 kb isoforms), but not glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*, loading control), in nL2 and nL8 as compared with their vector controls (neo-α and neo-σ).

To further confirm the stimulation of VEGF promoter activity by 12-LOX, we examined whether or not a transient increase in 12-LOX expression could stimulate VEGF promoter activity. Wild-type PC-3 cells were transiently transfected with a VEGF promoter construct and simultaneously, a 12-LOX expression construct or its vector control, pcDNA3.1. Co-transfection with the 12-LOX construct significantly increased 12-LOX expression (data not shown) and as shown in Fig. 4*B*, simultaneously, enhanced VEGF promoter activities, as compared with pcDNA3.1 vector control ($p < 0.05$). The results further confirmed the stimulation of VEGF promoter activity by 12-LOX.

The effects of 12(*S*)-HETE, the major eicosanoid product of 12-LOX, on VEGF promoter activity was then studied. Wild-type PC-3 cells were transfected with p1176 VEGF promoter construct and LacZ vectors and then treated with graded levels of 12(*S*)-HETE. As shown in Fig. 4*C*, after normalization for transfection efficiency, 12(*S*)-HETE increased VEGF promoter activity in PC-3 cells in a dose-dependent manner. The data suggest that 12(*S*)-HETE can modulate VEGF expression at transcriptional level in human prostate cancer cells. We also found that 12(*S*)-HETE treatment increased VEGF promoter activity in Du145 cells (data not shown).

Involvement of an Sp1/AP2 Cluster in 12-LOX-stimulated VEGF Expression—VEGF transcription in human cells is regulated primarily by the transcription factors Sp1, AP2, HIF-1, and AP1. In addition, NF-κB regulates mouse VEGF promoter activity in mouse cell lines.

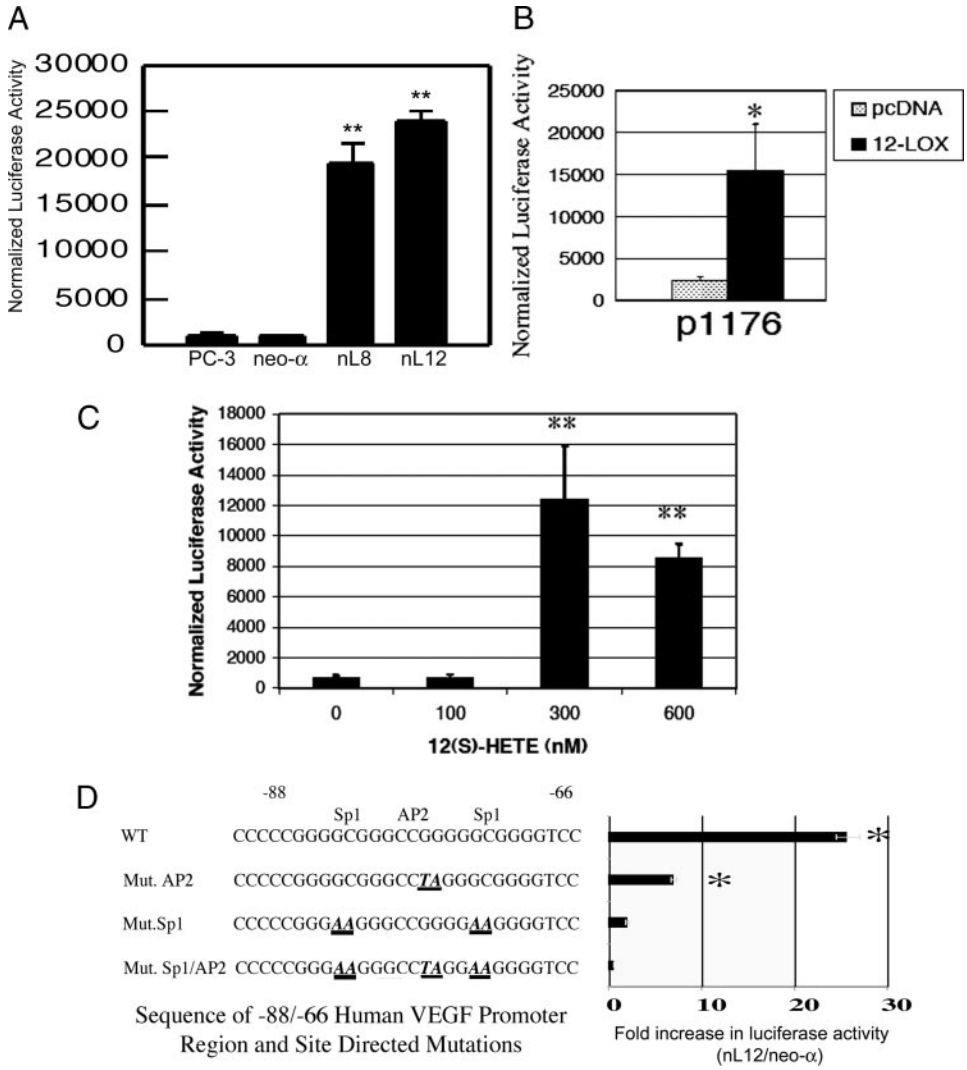

FIGURE 4. **Regulation of VEGF gene promoter activity by 12-LOX and 12(*S*)-HETE.** *A*, increased VEGF promoter activities in 12-LOX-transfected PC-3 cells. PC-3, neo-α, nL8, and nL12 cells were transfected with a VEGF promoter luciferase construct (−1176/+54) and a LacZ β-gal expression construct as described under "Experimental Procedures." Values are the average of normalized luciferase activities from three dishes ± S.D. **, $p < 0.01$ (Student's *t* test). *B*, increased VEGF promoter activity by transiently expressed 12-LOX in PC-3 cells. PC-3 cells were transiently transfected with VEGF promoter luciferase constructs (p1176, −1176/+54 promoter region), in the presence of a 12-LOX expression construct or its vector, pcDNA3.1. Luciferase activities were measured and normalized with β-gal activities as described under "Experimental Procedures." Values are the average from three dishes ± S.D. *, $p < 0.05$ (Student's *t* test). *C*, stimulation of VEGF promoter activity in PC-3 cells by 12(*S*)-HETE. PC-3 cells were transfected with p1176 VEGF promoter construct, along with a LacZ construct, and then treated with graded levels of 12(*S*)-HETE for 24 h. Shown here is a representative from three independent experiments. Values are the average of normalized luciferase activities from three dishes ± S.D. **, $p < 0.01$ (Student's *t* test). *D*, site-directed mutation analysis of the role of Sp1 and AP2 recognition sequences in 12-LOX-stimulated VEGF promoter activity. *Left panel*, DNA sequence between −88 and −66 of VEGF promoter. Mutated nucleotides in Sp1 recognition sequences (GC to AA) or AP2 binding site (GG to TA) are shown. *Right panel*, fold increase of luciferase activities in nL12 when compared with those in neo-α. *, $p < 0.05$, indicating the significant difference in luciferase activities between neo-α and nL12.

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These factors bind to their respective recognition sites present in the promoter region of the *VEGF* gene (16). Systematic sequence analysis of the VEGF promoter region revealed that the region from −66 to −88 encompasses a GC box, which serves as the binding site for the transcription factors Sp1 and AP2. This region has two Sp1 and one AP-2 binding site (16).

To identify whether this region plays a role in 12-LOX-induced stimulation of VEGF promoter activity, deletional mutants of the p1176-luc vector with (−88/+54) and without (−66/+54) the region between −66 and −88 were used. Normalized luciferase activity results clearly demonstrated that the presence of the region between −66 and −88 is necessary for the 12-LOX-mediated VEGF promoter activity, and deletion of this region resulted in loss of activity (data not shown). To identify whether Sp-1 or AP2 or both factors play a role in 12-LOX-mediated VEGF expression, VEGF promoter constructs (−88/+54) harboring site directed mutations targeting the binding sites for these two transcription factors were employed (19). The constructs had mutations in either both the Sp-1 binding sites or the AP2 binding site or all the three binding sites (Fig. 4*D*) These constructs were co-transfected separately with the LacZ plasmid into nL8 and neo cells and compared with activity generated upon transfection of the −88/+54 promoter construct lacking any mutation. As shown in Fig. 4*D*, mutation of the AP2 binding site decreased, but did not abolish, the stimulation of promoter activity by 12-LOX (from 25-fold to 8-fold increase). In contrast, mutation of the two Sp1 binding sites dramatically decreased the stimulation of VEGF promoter activity by 12-LOX in which the increase of VEGF promoter activity in nL8 cells was no longer statistically significant. These studies clearly demonstrate that the transcription factor Sp1 and to a lesser extent AP2 are involved in 12-LOX-mediated up-regulation of VEGF promoter activity and gene expression.

Signaling Pathways Linking 12-LOX Activity and VEGF Transcription—A variety of signaling pathways have been identified to be involved in the regulation of VEGF expression in cells (26). Activation of the PI 3-kinase pathway has been linked to VEGF expression in cancer cells (16). It was found that 12-LOX-overexpressing PC-3 cells showed an increase in PI 3-kinase activity as indicated by an almost 2-fold increase in the formation of PIP2 (Fig. 5*A*), which was inhibited by a PI 3-kinase inhibitor, LY294002 (20 μM). There also was a simultaneous increase in Akt phosphorylation in 12-LOX-transfected PC-3 cells (nL8) when compared with their vector control (neo-σ) (Fig. 5*B*, *left*). The increased Akt phosphorylation was reduced by treatment of nL8 cells with a specific 12-LOX inhibitor, baicalein (Fig. 5*B*, *right*). There was a 35% decrease in the level of p-Akt upon baicalein treatment based on densitometric analysis (data not shown). The data suggest that there is an increase in PI 3-kinase activity in PC-3 cells as a result of 12-LOX overexpression. To identify whether 12-LOX-mediated increase in the activity of PI 3-kinase and Akt is responsible for the increased VEGF expression, we treated the cells with LY294002, a chemical inhibitor of PI 3-kinase. As shown in Fig. 5*C*, LY294002 (20 μM) treatment led to a dramatic decrease in VEGF secretion in both 12-LOX-transfected PC-3 cells (nL8 and nL12) and neo-controls (neo-α and neo-σ), as well as the PC-3 parental cell line. Transfection with a dominant negative form of Akt also was found to reduce VEGF expression in 12-LOX-transfected PC-3 cells (data not shown). These findings clearly demonstrate that 12-LOX enzyme activity drives the up-regulation of *VEGF* gene expression via the PI3K-Akt signaling pathway in 12-LOX-overexpressing prostate cancer cells.

VEGF Secreted by 12-LOX-overexpressing PC-3 Cells as an Effector of Endothelial Cell Angiogenic Response—Overexpression of 12-LOX in PC-3 cells results in highly angiogenic tumors (11). To study whether

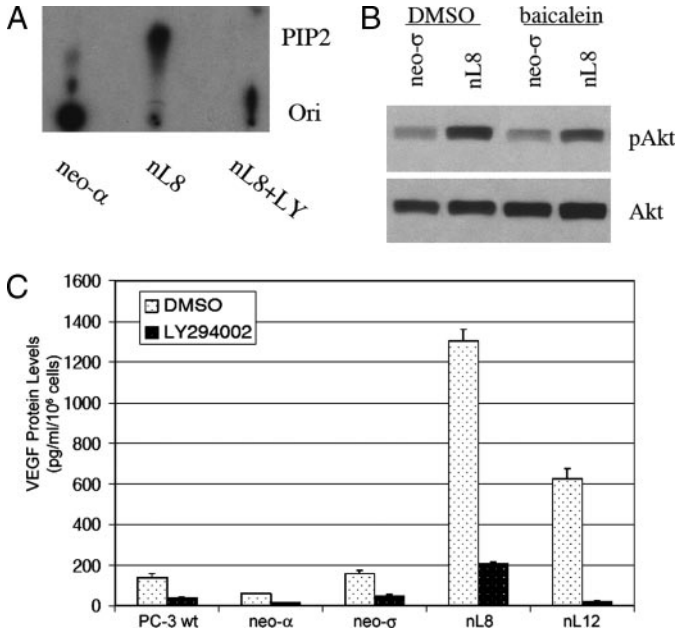

FIGURE 5. **Involvement of PI 3-kinase/Akt signaling pathway in VEGF expression.** *A*, increased PI 3-kinase activity in PC-3 cells by 12-LOX expression. PI 3-kinase activity in p85 immunoprecipitates was measured by incubating with substrate and [γ-^{32}P]ATP. Phospholipids were separated by TLC and autoradiographed. Shown here is a representative of three independent experiments. Note the increased PIP2 production in nL8 when compared with neo and its sensitivity to a PI 3-kinase inhibitor, LY294002 (*LY*). *Ori*, origin. *B*, increased Akt phosphorylation in 12-LOX-transfected PC-3 cells and its attenuation by baicalein. Neo-α and nL8 cells were treated with Me_2SO (*DMSO*) or 15 μM baicalein for 16 h. Cell lysates were harvested, and the phosphorylation of Akt was analyzed by using a polyclonal antibody against pSer^{473} Akt (*top panel*). *Bottom panel*, the membrane was stripped and reprobed for total Akt (phosphorylated and nonphosphorylated). *C*, reduction of VEGF expression by PI 3-kinase inhibitor LY294002. PC-3, neo-α, neo-σ, nL8, and nL12 cells were treated with 20 μM LY294002 or Me_2SO (*DMSO*) for 24 h. The VEGF protein levels in culture media were then measured by using ELISA as described under "Experimental Procedures," and the values were normalized to cell number. Values are means ± S.D. of three culture dishes. Shown here is a representative result from four independent experiments.

VEGF mediates the angiogenic activity of 12-LOX in prostate cancer cells, we evaluated the effect of neutralizing antibodies against VEGF or IL-8 on endothelial cell migration, as stimulated by the conditioned medium from 12-LOX-transfected PC-3 cells (nL8). As shown in Fig. 6, *A* and *B*, in the presence of control mouse IgG, the concentrated medium from 12-LOX-transfected PC-3 cells (nL8) stimulated endothelial cell migration ($p < 0.01$, Student's t test) when compared with those from vector controls (neo-θ). In the presence of neutralizing antibody against IL-8 as indicated by nIL8, the concentrated medium from 12-LOX-transfected PC-3 cells still retained the ability to stimulate endothelial cells ($p < 0.01$, Student's t test) (*bottom panel* in Fig. 6*A*; Fig. 6*B*). However, this increased ability was reduced to insignificant levels ($p > 0.05$) by pretreating the tumor cell-conditioned medium with a VEGF neutralizing antibody as indicated by nVEGF (*middle panel* in Fig. 6*A*; Fig. 6*B*). The data suggest that enhanced VEGF secretion from 12-LOX-overexpressing PC-3 cells can lead to an enhanced angiogenic response by stimulating endothelial cell migration.

DISCUSSION

The data presented in this study conclusively demonstrate that the enzyme 12-lipoxygenase and the metabolite 12(*S*)-HETE stimulate VEGF expression and secretion in prostate cancer cells. The results obtained from the experiments described in this work provide a strong basis for our previous observations of increased angiogenicity of PC3 cells overexpressing 12-LOX (11). By increasing VEGF secretion and in turn angiogenesis, 12-LOX may enhance the survival and promote

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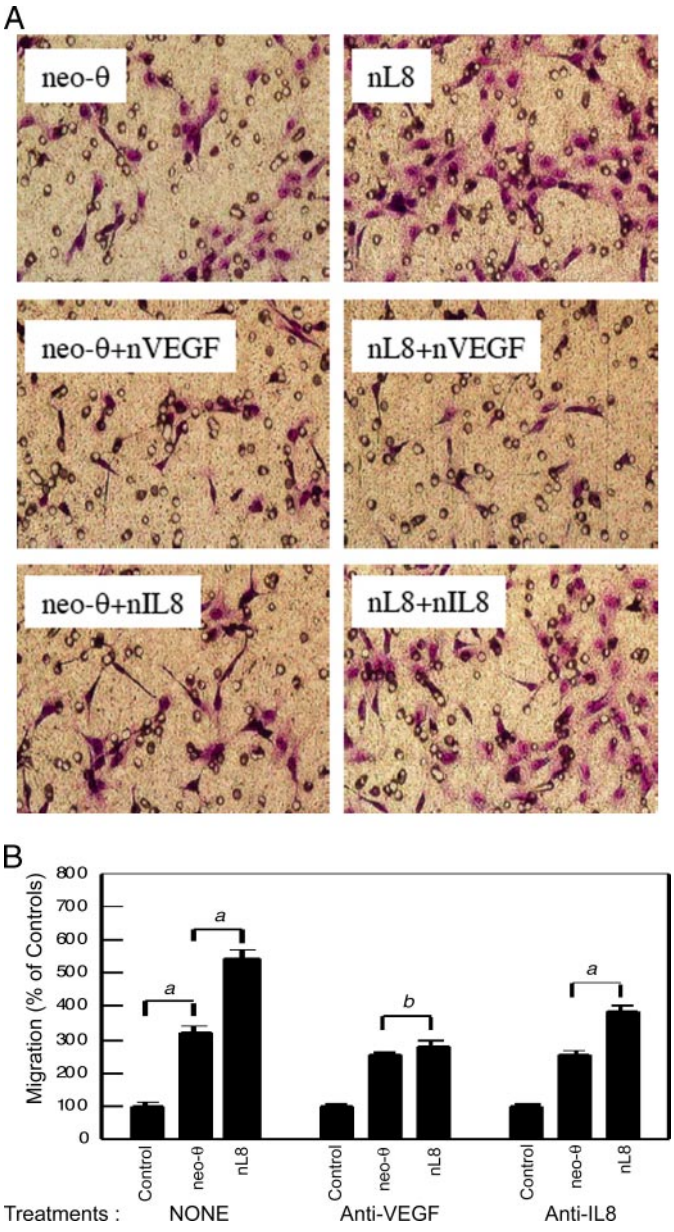

FIGURE 6. **Increased angiogenicity of 12-LOX-transfected PC-3 cells is partially mediated by VEGF.** *A*, stimulation of endothelial cell migration by nL8 is blocked by a VEGF neutralizing antibody but not by an IL-8-neutralizing antibody. Conditioned medium from 12-LOX-transfected PC-3 cells (nL8) and their vector controls (neo-θ) were harvested and concentrated 6 X using Centricon-10. HUVEC migration assay, in response to the conditioned medium, was conducted as described under "Experimental Procedures." Shown here are migrated endothelial cells (as indicated by *purple* or *dark purple staining*) in response to the conditioned medium. Note that the stimulation of endothelial cell migration by nL8 when compared with its vector control, neo-θ (*top panel*). This increased stimulation of endothelial cell migration was blocked by the pretreatment with a VEGF neutralizing antibody (as indicated by nVEGF) (*middle panel*), but not by an IL-8-neutralizing antibody (*bottom panel*). *B*, enumeration of endothelial cell migration. The migrated endothelial cells in response to different stimuli were counted in a double blind approach and the migration of endothelial cells was expressed as the percentage of the medium control in which no conditioned medium was added. *Column*, the average percentage of migration when compared with the medium control; *bar*, S.D. from six membranes. *a*, $p < 0.01$; *b*, $p > 0.05$ according to Student's *t* test.

metastasis of tumor cells from the primary site. Previous studies have shown that 12-LOX is a positive modulator of tumor cell survival and stimulates the activity of various anti-apoptotic factors in tumor cells (8–10, 27). The present observations have revealed that 12-LOX can potentially stimulate tumor cell survival and angiogenesis by modulating VEGF expression.

Tumor angiogenesis is a complex phenomenon mediated by a variety of growth factors, cells, and cell surface molecules in the tumor stromal microenvironment. Presently, a large number of clinical trials are aimed at inhibiting angiogenesis in cancers using novel therapeutic approaches. VEGF has been documented as a major angiogenic factor involved in the survival and metastasis of solid tumors, and its expression has been correlated with poor patient survival in many neoplastic disorders. VEGF expression and secretion are stimulated by a vast multitude of factors, which originate from genetic and physiological alterations, commonly found in cancer cells (13, 15, 28, 29). As a key cytokine in tumor angiogenesis, the expression of VEGF is tightly regulated at the transcriptional, post-transcriptional, and/or translational levels (15, 16). It has been extensively reported that the expression of VEGF is regulated by the activation of oncogenes such as Ras and Src, tumor suppressors such as P53, and physiological stressors such as hypoxia or hypoglycemia within the tumor microenvironment (16, 30).

Our studies suggest that 12-LOX is able to regulate *VEGF* gene expression at the transcriptional level. First, Northern blot analysis revealed an increase in the steady state levels of VEGF mRNA in 12-LOX-transfected PC-3 cells, indicating that there is an enhanced transcription or stabilization of VEGF mRNA. This finding was supported by the results from the RT-PCR analysis. Second, transfection with a VEGF promoter luciferase construct (−1176/+54) elicited a more than 10-fold increase in VEGF promoter activity in 12-LOX-transfected PC-3 cells as compared with their vector controls, suggesting that there was an increased VEGF promoter activity in PC-3 cells as a result of increased 12-LOX expression. Finally, transient cotransfection with a 12-LOX expression construct enhanced VEGF promoter activity in PC-3 cells. Although 12-LOX is able to stimulate VEGF expression at the transcriptional level, further studies are required to determine whether 12-LOX is involved in enhancing the stability or translation of VEGF mRNA.

Both NDGA and baicalein reduced VEGF expression, indicating that the enzymatic activity of 12-LOX plays a significant role in the regulation of *VEGF* gene expression. Exogenously added 12(*S*)-HETE was found to increase VEGF expression at both protein and promoter levels. It should be noted, however, that the extent of stimulation of VEGF expression by 12(*S*)-HETE is much less than that observed in PC-3 cells stably expressing 12-LOX. This may be caused by the inability of exogenously added 12(*S*)-HETE to mimic the cellular activities of endogenously produced 12(*S*)-HETE because of temporal and spatial limitations. PC-3 cells stably transfected with 12-LOX maintain a constant production of 12(*S*)-HETE. On the other hand, exogenously added 12(*S*)-HETE may be rapidly uptaken by cells and trapped in the phospholipid pool making it unavailable for biological responses. Thus external concentrations of 12(*S*)-HETE is greatly diminished for the treatment time of 48 h, as compared with the stable transfectants, which maintain a sustained production of the eicosanoid. Yet another reason for the lessened activity of exogenous 12(*S*)-HETE could be the fact that the majority of the biological activity of 12-LOX may be mediated by the immediate metabolite 12(*S*)-HPETE, and 12(*S*)-HETE the metabolite derived from degradation of 12(*S*)-HPETE contributes only a smaller percentage. Because 12(*S*)-HPETE is a short lived compound it is not practical to use this metabolite for exogenous cell treatment experiments. In addition to 12-LOX and 12(*S*)-HETE, other eicosanoids or eicosanoid producing enzymes have been found involved in the regulation of VEGF expression. For example, VEGF induction by cobalt chloride-simulated hypoxia is maintained by a concomitant, persistent induction of COX-2 expression and sustained elevation of prostaglandin E_2 synthesis in a human metastatic prostate cancer cell line (31).

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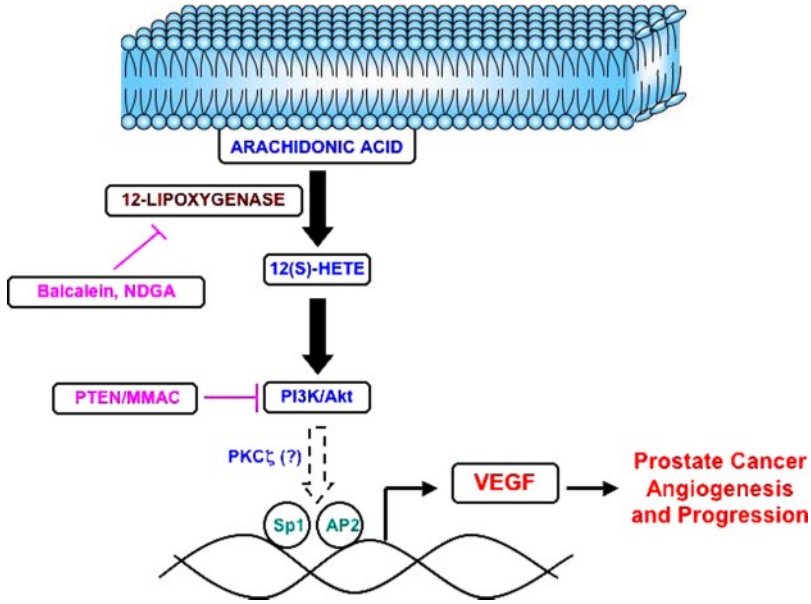

FIGURE 7. **Schematic representation of the proposed 12-LOX regulation of *VEGF* gene expression.** 12-LOX inserts oxygen into arachidonic acid to form 12(*S*)-HETE and reactive oxygen species, which can be inhibited by NDGA and baicalein. An increase in 12-LOX expression or activity leads to increased levels of 12(*S*)-HETE, which subsequently leads to the activation of PI 3-kinase. Activated PI 3-kinase produces $PI(3,4,5)P_3$, which serves as docking sites for Akt and the phosphatidylinositol-dependent kinase PDK. After binding to $PI(3,4,5)P_3$, Akt alters its conformation so that it can be phosphorylated and activated by PDK. Tumor suppressor PTEN can hydrolyze $PI(3,4,5)P_3$. In addition to Akt, PKCζ can also be regulated by PIP3. Activated PI 3-kinase/Akt signaling pathway results in increased *VEGF* gene transcription through the activation of *VEGF* gene regulatory proteins such as Sp1, which leads to enhanced transcription of the *VEGF* gene and production of VEGF protein.

Inhibition of COX-2 activity reduced hypoxia induced VEGF expression (31).

In addition to COX-2, 15-LOX-1 and 5-LOX of the lipoxygenase family are also implicated in the regulation of VEGF expression. Overexpression of 15-LOX-1 in PC-3 cells stimulates tumor growth via upregulation of VEGF (32). Inhibition of 5-LOX by MK886 reduces VEGF expression and decreases survival in malignant mesothelial cells (33). However, the mechanistic basis underlying eicosanoid regulation of VEGF expression has not been examined in detail. Here we present data for the first time that 12-LOX and 12(*S*)-HETE can regulate VEGF gene expression at transcriptional level. It will be interesting to see whether or not other eicosanoids regulate VEGF expression at the transcriptional level similar to 12-LOX or 12(*S*)-HETE.

We identified the PI 3-kinase pathway as an important signaling pathway for 12-LOX to increase VEGF expression in human prostate cancer cells as illustrated in Fig. 5. Increased 12-LOX expression or activity leads to the increased formation of 12(*S*)-HETE, which subsequently activates PI 3-kinase (34). Increased PI 3-kinase activity was observed in 12-LOX-transfected PC-3 cells. In congruence with the observed increase in PI 3-kinase, we found an increase in the activation of Akt, a downstream effector of PI 3-kinase, in 12-LOX-transfected PC-3 cells as measured by the level of phosphorylation of Ser^{473} in Akt. Further the increased level of active Akt in 12-LOX-transfected PC-3 cells was attenuated by baicalein, a specific inhibitor of 12-LOX. Activated PI 3-kinase stimulates *VEGF* gene expression through the activation of its various downstream effectors. It has been reported that PI 3-kinase is required for hypoxia-induced VEGF expression in H-ras-transformed NIH3T3 cells (35). In prostate cancer, in addition to its role in cell survival in LNCaP cells (36), PI 3-kinase/Akt signaling pathway has been found to regulate VEGF expression through the expression or activation of hypoxia inducible factor 1α (37). In this study, it was found that inhibition of PI 3-kinase with a pharmacological inhibitor LY294002 or by a dominant negative inhibitor of Akt reduced *VEGF* gene expression. One of the downstream effectors of PI 3-kinase, Akt, was found to be involved in stimulation of VEGF expression by 12-LOX. The results suggest that the PI 3-kinase/Akt signaling pathway is involved in the stimulation of VEGF expression by 12-LOX. In addition to Akt, other downstream effectors of PI 3-kinase such as PKCζ are known to be activated by PIP3 and PDK (38, 39). PKCζ has been shown to regulate VEGF gene expression by interacting with the transcription factor Sp1 in renal carcinoma cells (40). Experiments are ongoing to determine whether PKCζ is activated by PI 3-kinase in prostate cancer cells and whether PKCζ activity is increased in 12-LOX-transfected PC-3 cells and if so, what role PKCζ plays in increased VEGF expression.

Finally it is worthwhile to note that the cellular signaling from PI 3-kinase can be attenuated by PTEN/MMAC1 tumor suppressor protein, a lipid phosphatase that functions as a negative regulator of the PI 3-kinase/Akt pathway (41). In primary prostate cancer, loss of PTEN expression correlates with high Gleason score and advanced stage (42, 43). Inactivation of PTEN tumor suppressor gene has been found associated with increased angiogenesis in localized prostate carcinoma (44). It remains to be determined, however, whether PTEN attenuates 12-LOX stimulation of prostate cancer angiogenesis and progression and whether inactivation of PTEN or loss of PTEN expression augment the stimulation of tumor angiogenesis by 12-LOX. The above discussion is summarized in Fig. 7.

In summary, the present study describes a novel link between 12-LOX and VEGF expression in prostate cancer cells. We demonstrated VEGF as an important effector in 12-LOX-enhanced angiogenicity of prostate tumor cells. We further showed an important role for PI 3-kinase-dependent signaling pathway in 12-LOX-stimulated *VEGF* gene expression. The findings reported herein provide a novel insight into how eicosanoids modulate tumor angiogenesis and progression.

Acknowledgments—Performance of this work was facilitated by the Microarray and Bioinformatic Facility Core of the Environmental Health Sciences Center in Molecular and Cellular Toxicology with Human Applications at Wayne State University.

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